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Original Article

In Situ Biosynthesis of Natural Fruity Flavors in Whey and Whey Permeate during Fermentation Using Lipase

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ABSTRACT

Background and Objectives: Chemical synthesis and extraction of flavorings from natural sources include disadvantages. However, biotechnology is a preferred method for biosynthesis of flavorings. The objective of this study was to synthesize natural esters with fruity flavors in whey using lipase of Palatase in combination with ethanol fermentation.

Materials and Methods: *Lactobacillus fermentum* ATCC 14931, *Lactobacillus reuteri* DSM 17938, and *Lactobacillus buchneri* NCIMB 40788 were used as *in situ* ethanol producers. Ultra-high temperature (UHT) cream (30% fat) was added to the fermentation media as a fat source, which was esterified with produced ethanol by Palatase. Viable cell counts and pH were monitored at 0, 24 (Palatase addition), and 48 h (end of fermentation). Free fatty acids of up to 12 carbon chain and their ethyl esters were analyzed using gas chromatography/mass spectrometry.

Results: Palatase did not affect the viable cell counts of *Lactobacillus reuteri* and *Lactobacillus buchneri*, but increased the viable cell counts of *Lactobacillus fermentum*. Ethyl ester and free fatty acid levels of the samples increased after Palatase addition. *Lactobacillus fermentum* produced higher ester levels in ultrafiltration whey permeate whereas other lactobacilli produced higher ester levels in traditional whey. *Lactobacillus buchneri* included the lowest ester levels and *Lactobacillus reuteri* included the lowest free fatty acid levels between the lactobacilli. The ester levels of *Lactobacillus reuteri* samples were the highest in most cases. Free fatty acid levels were higher in traditional whey than ultrafiltration whey permeate.

Conclusions: This method can be recommended as an alternative to artificial flavorings with the advantage of labeling products as natural.

Keywords: Fermentation, Flavor ester, *Lactobacillus*, Lipase, Whey

Introduction

Flavor is one of the most important factors in the food industry since it affects product acceptance in the market (1). Although flavor compounds are naturally detected in foods, they are added to food products as flavor enhancers or improvers (2). Food flavorings are divided into three major categories of natural, nature-identical and artificial flavorings. Based on the consumer demands, production and use of natural flavors replace those of chemically synthesized flavors. Natural flavors are flavors that are produced from natural materials using extraction and biotechnological methods. Since extraction is expensive, biotechnology is the preferred method of producing these compounds. Microbial fermentation and enzyme use are two common biotechnological methods in flavor production. Naturally, efficiency of the enzymatic method is higher than that of fermentation method (3, 4). A wide range of chemical compounds such as esters can attribute to food flavors (5). Esters of short and medium chain fatty acids (SCFA and MCFA, respectively) and alcohols are used in food industries as flavor compounds with pleasant fruity flavors. However, esters of fatty acids (FA) with carbon chains longer than 12 cause undesirable soapy or tallowy tastes (6). Lipases are biocatalysts with the ability of producing esters via interesterification, alcoholysis and

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direct esterification of FAs with alcohols. Although, most lipases include catalytic activities in media with low water activity, a few lipases can produce esters in aqueous media (7). Of these lipases, a purified lipase from *Rhizomucor miehei* produced by the recombinant *Aspergillus oryzae* with the commercial name of Palatase 20000 L has been introduced for use in foods (8). Palatase specificity uses short chain fatty acids (SCFA) for esterifying and hence does not leave undesirable flavors (7). This food-grade enzyme has been used for ester productions in foods (6, 9, 10).

One of the methods that have recently been developed to produce fruity flavors in foods includes *in situ* ester production via enzymatic biosynthesis (7, 9–11). Alcohol is added manually in most *in situ* ester productions; however, researchers benefit microbial fermentation to eliminate alcohol handling which seems a better alternative. Liu et al. (2009) and Zhang et al. (2016) used spiked ethanol to produce flavor esters in dairy products (10, 11). However, combination of fermentation with lipase addition to produce fruity flavor in coconut creams has been carried out in a few studies using *in situ* produced ethanol. Triglycerides (TG) of coconut creams were esterified with produced ethanol using Palatase. Labeling food products as natural is an advantage of *in situ* ester production using the combined systems (9). The worldwide production of whey is estimated as nearly 120 million tons annually, which raises environmental concerns. Researchers try to transform whey into value-added products; however, large quantities of whey are still discarded into the environments of developing countries (12, 13). Heterofermentative lactic acid bacteria (LAB) include abilities to produce ethanol and esters, which are important factors in development of flavors (14, 15). However, their use as ethanol producers with Palatase in ester synthesis has not been reported yet. *Lactobacillus* is one of the important genera of LAB with homo and heterofermentative species (16). The major objective of this study was to assess *in situ* biosynthesis of natural ethyl esters with fruity flavors in traditional whey and ultrafilter (UF) whey permeate using lipase Palatase in combination with alcoholic fermentation. Three heterofermentative lactobacilli were used as ethanol producing cultures. The FAs, the other precursor of ester synthesis, were produced by adding milk fat (cream) to the whey media. Enumeration of the bacterial viable cell and pH measurement were carried out to monitor the fermentation process. Since sensory analysis normally depends on individuals, a further precise method, gas chromatography/mass spectrometry (GC/MS), was used to analyze the produced esters as well as FFAs.

Materials and Methods

Materials and microorganisms

In general, de Man Rogosa Sharpe (MRS) broth, MRS agar, bacteriological peptone and hexane were purchased from Merck, Darmstadt, Germany. Lipase of Palatase 20000 L (Novozymes, Bagsværd, Denmark) and Chymosin CHY-MAX (Chr. Hansen A/S, Hørsholm, Denmark) were purchased with high grades. *Lactobacillus fermentum* ATCC 14931 (PTCC 1744) was purchased from Persian Type Culture Collection, Tehran, Iran, as pure culture in freeze-dried form. *Lactobacillus reuteri* DSM 17938 and *Lactobacillus buchneri* NCIMB 40788 were respectively isolated from Biogia and Lalsil fresh in Food Microbiology Lab of the University of Shiraz, Shiraz, Iran. Permeate powder of UF cheese whey (95%, w/w dry matter), pasteurized milk (1.5% fat) and ultra-high temperature (UHT) cream (30% fat) were purchased from Pegah Fars Dairy Industry, Shiraz, Iran.

Preparation of fermentation media

The UF whey permeate powder was dissolved in distilled water (DW) to prepare UF whey permeate solution with 5% dry matter. Cheese curd was prepared by heating pasteurized milk to 45 °C and adding 0.4% of chymosin. Cheese curd was filtered to prepare traditional whey. The UF whey permeate and traditional whey were sterilized using autoclave. Moreover, UHT cream was added to two fermentation media at 10% to provide FA source for ester production.

Preparation of microbial inocula

Lactobacillus cultures were propagated in MRS broth at 37 °C for 48 h and stored at -80 °C as stock cultures. Starter cultures for fermentation were prepared by transferring 5% (v/v) of thawed stock cultures into fresh MRS broth and incubating at 37 °C for 24 h. After two consecutive transfers of the microbial cultures, centrifuging at 8000 g for 10 min at 4 °C was carried out to harvest cells, followed by washing cell pellets twice with physiological saline (8.5 g L^{-1} NaCl). Then, washed cells were resuspended in fresh saline with a similar volume prior to inoculation into fermentation media (17).

Fermentation in presence of lipase (Palatase)

Briefly, UF whey permeate solution and traditional whey were used as fermentation media. The two media were inoculated with the prepared lactobacilli inocula at 1% (v/v) as mono-cultures. Inoculated media were dispensed in 30 mL aliquots in 50-mL polypropylene tubes and incubated at 30 °C for 48 h. Lipase (undiluted and filter sterilized) was added into inoculated tubes at 0.1% (v/v) concentrations after 24 h of incubation. Sampling from the tubes was carried out at 0, 24, and 48 h time intervals to enumerate microbial cells and measure pH of the samples.

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Inoculated media with no added Palatase and uninoculated media with 0.1% Palatase served as controls. Appropriate Palatase concentration as well as fermentation duration time were reported based on preliminary experiences. Enumeration of viable cells was carried out as duplicate using MRS agar plates. Inoculated MRS agars were incubated at 37 °C until colonies grew (24–48 h). The pH measurement was carried out using calibrated pH meter (GLP 22, Crison, Barcelona, Spain).

Ester and free fatty acid analyses by gas chromatography/mass spectrometry

Fermented and control samples of 48 h fermentation as well as media with no fermentation and Palatase addition were analyzed for even-numbered free fatty acids (FFA) (up to 12 carbon chain) and their ethyl esters. Samples were prepared by addition of 3 mL of hexane to 5 mL of sample and vortexing to extract esters and FAs into hexane phase. Gas chromatograph (HP 6890N, Hewlett Packard, Palo Alto, CA, USA) equipped with DB-225ms capillary column (30 m length, 250 µm i.d., 0.25 µm film thickness) (Agilent Technologies, Wilmington, DE, USA) was used. Helium with a flow rate of 24 mL min^{-1} was used as carrier gas and Agilent 5973N Inert Mass Selective Detector (MSD) was used for detection. The MS detector was operated in electron ionization mode (70 eV) with ion source temperature of 230 °C. The temperature was held at 50 °C for 1 min, followed by an increase to 220 °C by 5 °C min^{-1}, holding at this temperature for 5 min. Samples were injected in 1-µL volumes at a split ratio of 1:20 at 280 °C. Esters and FFAs were identified based on the US National Institute of Standards and Technology and Wiley Libraries databases. Verification of the identified compounds was carried out by the comparison of their retention times with those of C9–C17 saturated alkane standards.

Statistical analysis

Data were reported as mean values ± SD (standard deviation) of three independent experiments (n = 3). One-way analysis of variance (ANOVA), Duncan's multiple range test and SPSS Software v.16.0 (IBM Analytics, USA) were used to show significant differences at $p < 0.05$.

Results

Fermentation in presence of lipase (Palatase)

Table 1 demonstrates effects of Palatase addition on the viable cell counts of three *Lactobacillus* cultures during a 48-h fermentation period in UF whey permeate and traditional whey. Table 2 displays respective pH values of the corresponding samples. Accordingly, samples with similar *Lactobacillus* cultures included similar viable cell counts and pH values at the beginning of fermentation. Furthermore, each microbial culture included similar cell counts and pH values during fermentation before the Palatase addition (0 and 24 h). This was expected as lactobacilli were inoculated in similar quantities into the samples. Based on Table 1, addition of Palatase to fermentation media at Hour 24 did not significantly affect the growth rate and final viable cell counts of *L. reuteri* and *L. buchneri*. However, increases of 0.51 and 0.65 log CFU/mL were seen in viable cell counts of *L. fermentum* after Palatase addition to UF whey permeate and traditional whey, respectively. After 48 h of fermentation, no significant differences were seen in the viable cell count of each *Lactobacillus* between the UF whey permeate and the traditional whey. Based on Table 2, *L. reuteri* did not show significant differences in pH values between the control and Palatase treated samples. For *L. buchneri* and *L. fermentum*, pH decreased in UF whey permeate samples after 48 h; however, pH did not change in traditional whey samples.

Table 1. Changes in microbial counts (log CFU/mL) during 48-h fermentation of traditional whey and UF whey permeate by *Lactobacillus* species using Palatase

Fermentation medium	Time (h)	With Palatase			Without Palatase		
		Lactobacillus fermentum	*Lactobacillus buchneri*	*Lactobacillus reuteri*	*Lactobacillus fermentum*	*Lactobacillus buchneri*	*Lactobacillus reuteri*
UF whey permeate	0	7.05 ± 0.12^{a}*	7.17 ± 0.11^{a}	7.17 ± 0.11^{a}	7.01 ± 0.16^{a}	7.17 ± 0.11^{a}	7.13 ± 0.13^{a}
	24	7.48 ±0.07^{b}	7.22 ± 0.01^{a}	8.18 ± 0.06^{b}	7.55 ± 0.11^{b,c}	7.09 ± 0.12^{a}	8.14 ± 0.06^{b}
	48	8.22 ± 0.07^{d}	8.12 ± 0.08^{b}	8.42 ± 0.05^{c,d}	7.71 ± 0.27^{b,c}	8.25 ± 0.02^{b}	8.47 ± 0.09^{d}
Traditional whey	0	7.05 ± 0.11^{a}	7.20 ± 0.15^{a}	7.14 ± 0.18^{a}	7.07 ± 0.11^{a}	7.20 ± 0.17^{a}	7.15 ± 0.15^{a}
	24	7.74 ± 0.05^{c}	7.11 ± 0.13^{a}	8.27 ± 0.14^{b,c}	7.73 ± 0.02^{b,c}	7.20 ± 0.05^{a}	8.26 ± 0.07^{b,c}
	48	8.29 ± 0.17^{d}	8.11 ± 0.20^{b}	8.47 ± 0.13^{d}	7.64 ± .032^{b,c}	8.10 ± 0.22^{b}	8.47 ± 0.16^{d}

* Each microorganism analyzed separately. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the columns of each microorganism (with and without Palatase) with different lowercase letters are significantly different ($P < 0.05$).

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Table 2. Changes in pH values during 48-h fermentation of traditional whey and UF whey permeate by *Lactobacillus* species using Palatase

Fermentation medium	Time (h)	With Palatase			Without Palatase		
		Lactobacillus fermentum	*Lactobacillus buchneri*	*Lactobacillus reuteri*	*Lactobacillus fermentum*	*Lactobacillus buchneri*	*Lactobacillus reuteri*
UF whey permeate	0	6.46 ± 0.16^{d}*	6.45 ± 0.12^{c}	6.42 ± 0.13^{c}	6.46 ± 0.15^{d}	6.44 ± 0.15^{c}	6.42 ± 0.16^{c}
	24	5.76 ±0.40^{c}	6.31 ± 0.16^{c}	5.67 ± 0.37^{b}	5.78 ± 0.34^{c}	6.27 ± 0.21^{c}	5.76 ± 0.29^{b}
	48	4.57 ±0.06^{a}	4.99 ± 0.26^{a}	4.02 ± 0.34^{a}	5.12 ± 0.35^{b}	5.27 ± 0.15^{b}	4.51 ± 0.32^{a}
Traditional whey	0	6.43 ± 0.21^{d}	6.40 ± 0.19^{c}	6.42 ± 0.21^{c}	6.42 ± 0.21^{d}	6.38 ± 0.19^{c}	6.39 ± 0.23^{c}
	24	5.73 ± 0.34^{c}	6.26 ± 0.27^{c}	5.56 ± 0.33^{b}	5.76 ± 0.27^{c}	6.35 ± 0.03^{c}	5.54 ± 0.30^{b}
	48	4.73 ± 0.15^{a,b}	5.32 ± 0.01^{b}	4.06 ± 0.38^{a}	5.02 ± 0.33^{a,b}	5.33 ± 0.25^{b}	4.44 ± 0.41^{a}

* Each microorganism analyzed separately. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the columns of each microorganism (with and without Palatase) with different lowercase letters are significantly different (P < 0.05).

Ester and free fatty acid analyses using gas chromatography/mass spectrometry

In this study, ethyl esters of SCFAs (up to 12 carbon chains) were assessed. Results from Table 3 show the peak areas (×10^{5}) of FA ethyl esters from UF whey permeate and traditional whey after 48 h of fermentation with and without Palatase addition. No esters were detected in samples with no bacteria and Palatase. As shown in Table 3, ethyl acetate was the only ethyl ester detected in fermented samples with no Palatase. The *L. reuteri* with no Palatase addition produced no esters in UF whey permeate and traditional whey. Palatase addition significantly increased levels of all ethyl esters in all samples and samples containing esters smelled fruity. Based on Table 3, *L. buchneri* produced the lowest levels of esters in the two fermentation media, compared to that *L. fermentum* and *L. reuteri* did. In most cases, ester levels produced by *L. reuteri* were significantly higher than those produced by *L. fermentum*. However, *L. fermentum* was a more efficient ethyl acetate producer, especially in UF whey permeate media with a high peak area (×10^{5}) of 103.55. Despite *L. fermentum* with a higher ester level production in UF whey permeate, two other lactobacilli produced higher ester levels in traditional whey. Based on Table 4, decanoic and dodecanoic acids were detected with no significant differences in their levels in samples with no Palatase addition. Shorter chain free fatty acids (SCFFA) were not detected in these samples. However, Palatase addition increased FFAs in all treated samples. The *L. buchneri* samples containing Palatase included higher FFA levels than *L. reuteri* and *L. fermentum* samples in the two media. The *L. reuteri* samples included the lowest FFAs between the three lactobacilli strains. Palatase treated traditional whey samples fermented by *L. fermentum* included high FFA levels. Overall, FFA levels were higher in traditional whey rather than UF whey permeate.

Table 3. Ester levels (peak area ×10^{5}) of the samples after 48 h of fermentation by *Lactobacillus* species using Palatase

Fermentation medium & Palatase addition	Microorganism	Ethyl esters					
		Ethyl acetate	Ethyl butanoate	Ethyl hexanoate	Ethyl octanoate	Ethyl decanoate	Ethyl dodecanoate
UF whey permeate with Palatase	*Lactobacillus fermentum*	103.55 ± 27.33^{e}*	23.60 ± 4.28^{d}	26.30 ± 5.98^{d}	8.73 ± 3.13^{c}	21.04 ± 6.04^{c}	18.36 ± 4.39^{d}
	Lactobacillus buchneri	11.16 ± 2.91^{c,d}	2.11 ± 0.78^{a}	1.47 ± 0.72^{a}	nd**	nd	2.52 ± 0.60^{a}
	Lactobacillus reuteri	7.81 ± 2.20^{b}	24.07 ± 6.21^{d}	28.96 ± 7.85^{e}	8.35 ± 2.55^{b,c}	23.49 ± 5.81^{d}	23.53 ± 7.78^{e}
UF whey permeate without Palatase	*Lactobacillus fermentum*	5.34 ± 1.36^{a,b}	nd	nd	nd	nd	nd
	Lactobacillus buchneri	5.18 ± 1.83^{a,b}	nd	nd	nd	nd	nd
	Lactobacillus reuteri	nd	nd	nd	nd	nd	nd
Traditional whey with Palatase	*Lactobacillus fermentum*	12.29 ± 3.33^{d}	18.84 ± 5.11^{c}	20.64 ± 7.34^{c}	7.53 ± 1.81^{b}	18.16 ± 4.14^{b}	14.66 ± 4.05^{c}
	Lactobacillus buchneri	8.40 ± 2.03^{b,c}	8.16 ± 2.19^{b}	8.18 ± 2.70^{b}	2.63 ± 0.55^{a}	5.73 ± 1.59^{a}	5.28 ± 2.21^{b}
	Lactobacillus reuteri	6.19 ± 1.78^{a,b}	26.22 ± 7.63^{e}	42.00 ± 11.87^{f}	16.97 ± 3.08^{d}	38.87 ± 10.03^{e}	36.75 ± 11.38^{f}
Traditional whey without Palatase	*Lactobacillus fermentum*	4.37 ± 0.98^{a}	nd	nd	nd	nd	nd
	Lactobacillus buchneri	nd	nd	nd	nd	nd	nd
	Lactobacillus reuteri	nd	nd	nd	nd	nd	nd

* Each ester analyzed separately. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same column with different lowercase letters are significantly different (P < 0.05).
** Not detected

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Table 4. Free fatty acid production (peak area $\times 10^5$) in samples after 48 h of fermentation by *Lactobacillus* species using Palatase

Fermentation medium & Palatase addition	Microorganism	Fatty acids					
		Acetic acid	Butanoic acid	Hexanoic acid	Octanoic acid	Decanoic acid	Dodecanoic acid
UF whey permeate with Palatase	*Lactobacillus fermentum*	1.09 ± 0.33[a]*	16.91 ± 4.98[a]	48.19 ± 12.81[c]	38.27 ± 9.54[d]	69.46 ± 21.79[d]	66.36 ± 20.63[e]
	Lactobacillus buchneri	nd**	21.70 ± 5.51[b]	64.38 ± 13.19[d]	47.50 ± 13.80[e]	90.00 ± 21.43[e]	72.53 ± 22.58[f]
	Lactobacillus reuteri	1.67 ± 0.81[b]	16.09 ± 3.97[a]	34.88 ± 9.58[a]	28.44 ± 6.59[b]	42.71 ± 12.82[b]	26.78 ± 10.54[b]
UF whey permeate without Palatase	*Lactobacillus fermentum*	nd	nd	nd	2.55 ± 0.79[a]	4.47 ± 1.52[a]	3.79 ± 1.16[a]
	Lactobacillus buchneri	nd	nd	nd	nd	2.69 ± 0.66[a]	5.05 ± 2.01[a]
	Lactobacillus reuteri	nd	nd	nd	nd	3.88 ± 1.35[a]	1.61 ± 0.71[a]
Traditional whey with Palatase	*Lactobacillus fermentum*	nd	21.50 ± 6.86[b]	68.91 ± 17.07[d,e]	48.59 ± 13.13[e]	95.72 ± 27.25[f]	79.64 ± 21.62[g]
	Lactobacillus buchneri	nd	21.80 ± 7.13[b]	72.47 ± 13.56[e]	50.36 ± 18.21[e]	90.63 ± 26.90[e]	61.23 ± 18.57[d]
	Lactobacillus reuteri	2.42 ± 1.11[c]	20.94 ± 5.35[b]	40.01 ± 11.61[b]	32.65 ± 8.05[c]	55.35 ± 16.65[c]	38.11 ± 10.58[c]
Traditional whey without Palatase	*Lactobacillus fermentum*	nd	nd	nd	nd	2.75 ± 1.63[a]	1.18 ± 0.34[a]
	Lactobacillus buchneri	nd	nd	nd	nd	3.38 ± 1.56[a]	2.91 ± 0.95[a]
	Lactobacillus reuteri	nd	nd	nd	nd	0.99 ± 0.14[a]	1.80 ± 0.66[a]

* Each fatty acid analyzed separately. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same column with different lowercase letters are significantly different ($P < 0.05$).
** Not detected

Discussion

Based on the obtained results, increased viable cell counts of Palatase-treated *L. fermentum* samples was unexpected since the MCFAs released from fats due to hydrolytic activities of Palatase could include antibacterial effects (18). However, FFAs could not inhibit the growth rate of the selected lactobacilli in the current study. In a previous study by Sun et al. on *Saccharomyces cerevisiae* fermentation in coconut creams, yeast counts decreased after Palatase addition (9). This difference could be due to different stress tolerance levels of yeast and lactobacilli cells. Teichoic acid of the bacterial cell walls has been reported to protect bacteria against FFAs (19). The hydrolytic activity of Palatase was the reason of pH decrease in Palatase treated samples. It seemed that Palatase did not significantly decrease pH values in most of the samples, especially in traditional whey. This could be a result of esterification/transesterification reactions of Palatase, which decreased the FFA content of the media. Quantities of the esters produced by Palatase could be affected by the ethanol content produced during fermentation as well as viable cell counts of the ethanol producing microbial cultures and pH of the fermentation media. The working pH range of Palatase is naturally 5.0–9.5 with optimum of 6.5–7.5 (20). The viable cell count of *L. buchneri* was not lower than those of other *Lactobacillus* cultures within 48 h. The pH values of the samples fermented by *L. buchneri* were ≥ 5, which favored Palatase activity. Hence, lower ability of ethanol production was likely the reason of lower ester levels produced by *L. buchneri*. Ethyl acetate detected in fermented samples with no Palatase was not produced by the Palatase activity but produced by fermentation (21, 22). Based on the cell counts and pH values, ethanol contents of the media could be the major cause of higher ester productions in *L. reuteri* samples, compared to *L. fermentum* samples.

In UF whey permeate fermented by *L. fermentum*, factors other than esterification reactions seemed to be effective in ethyl acetate production. One possibility could be the released components due to hydrolytic activity of Palatase, which promoted ethyl acetate production by the bacteria. Lactic acid (LA) produced by lactobacilli species during fermentation (23) was expected to be esterified with ethanol in presence of Palatase. Nevertheless, ethyl lactate was not detected in the current study. This was most possibly due to lipase type and ability of Palatase in esterifying ethanol and LA. Ethanol and LA are naturally hydrophilic compounds, which prefer to set in the aqueous phase rather than the oil-water interface. This causes difficulties in direct esterification of the chemicals for Palatase (24). However, Palatase in its immobilized form could synthesize ethyl lactate in a previous study (25). In a study by Liu et al., Palatase could not directly esterify butanoic acid and ethanol whereas alcoholysis of tributyrin with ethanol was a better choice to synthesize ethyl butanoate by this lipase. This was due to the higher hydrophobicity of tributyrin (8). In fact, alcohol to acid ratio is an important factor in esterification reactions (26, 27). In the present study, ethanol level was not possibly sufficient for the esterification of LA because ethanol was gradually used by Palatase, while LA increased due to fermentation.

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Hydrophobic nature of the fermentation media is another effective factor in Palatase activity. Previously, Sun and Liu compared the mechanism of ester synthesis in coconut creams and buffer systems and revealed that the coconut cream media were further appropriate due to their higher hydrophobicity (24). Therefore, fat content added to whey could directly affect the produced ester levels. Other substances in the media could affect distribution of water between lipase and reaction media, affecting lipase activity as a result (24). Moreover, Palatase addition increased FFA levels due to its hydrolytic activity. The higher FFA levels of *L. buchneri* samples with Palatase addition were linked to its lower capacity in ethanol production, which caused non-esterified FFAs. In contrast, lower FFA levels could be due to higher esterification reactions in *L. reuteri* samples. In a previous study on coconut creams, it was reported that synthetic activity of Palatase was affected by pH whereas its hydrolytic activity was not affected (9). In the current study, the lower pH values of *L. reuteri* samples were not likely the reasons for low FA levels. Decanoic and dodecanoic acids in samples with no Palatase showed that these FAs were either products of fermentation (28) or releases from the fats of media by microbial esterases and lipases (29). Limitations of the GC method or low concentrations of SCFAs possibly caused them to be undetectable in samples with no Palatase (30). Totally, FFA levels were higher in traditional whey than UF whey permeate. One hypothesis includes various compositions of the two media with stimulatory or inhibitory effects on Palatase activity. It has been reported that various cations can stimulate or inhibit lipases (31). Inhibitory effects of immunoglobulins on lipases have been reported (32). However, fat contents and overall pH levels of the two media were similar and hence cannot be acceptable reasons. Despite smelling fruity and having potential to be consumed directly as a beverage with natural fruity flavorings, further studies are needed to improve tastes of the products. Low fat dairies can benefit these products in their formulations to improve their tastes (10). Esters include capabilities to mask goat-like and mutton-like flavors in dairy products (6).

Conclusion

This study was carried out to assess *in situ* fruity flavor biosynthesis in whey and UF whey permeate using lipase of Palatase with *Lactobacillus* fermentation. Overall, Palatase did not affect the growth rate of *Lactobacillus* cultures (except *L. fermentum*) with increase in viable cell counts of UF whey permeate and traditional whey media. No differences were reported in viable cell counts of *Lactobacillus* cultures between the UF whey permeate and traditional whey. Palatase decreased pH values of the fermented samples. Furthermore, Palatase addition increased ethyl ester levels of all the treated samples, generating fruity flavors. The *L. reuteri* was the most efficient ester producer while the lowest ester levels detected in *L. buchneri* samples. This is a novel method with advantages of using no synthetic flavorings as well as labeling products as natural. Products include potential to be consumed directly or as flavorings in food formulations. Although ethanol was the only alcohol used in this study, further studies can benefit the microorganisms to produce other alcohols to synthetize various esters. Addition of amino acids to fermentation media can be used to achieve this goal.

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